SureSelect Target Enrichment for Illumina Paired-End mRNA-Seq Library Prep

SureSelect RNA Capture for Illumina Paired-End Multiplexed Sequencing

Protocol

Version 1.1, May 2011

SureSelect platform manufactured with Agilent SurePrint Technology

Research Use Only. Not for use in Diagnostic Procedures.

Agilent Technologies

Notices

Manual Part Number

G7580-90010

Edition

Version 1.1, May 2011

Printed in USA

Agilent Technologies, Inc.
5301 Stevens Creek Rd
Santa Clara, CA 95051 USA

Acknowledgement

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CAUTION

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In this Guide...

This guide describes Agilent's recommended operational procedures to capture transcripts of interest using Agilent's SureSelect RNA Capture Kit and sample preparation kits for next-generation sequencing. This protocol is specifically developed and optimized to use Biotinylated RNA oligomer libraries, or Bait, to enrich targeted regions of the transcriptome.

This guide uses the Illumina paired-end multiplex sequencing platform for library preparation.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation

This chapter describes the steps to prepare the RNA sample for target enrichment.

3 Hybridization

This chapter describes the steps to prepare and hybridize samples.

4 Addition of Index Tags by Post-Hybridization Amplification

This chapter describes the steps to amplify, purify, and assess quality of the sample library.

What's New in Version 1.1

- Reagent cap colors are listed where available.
- More details given for the reagent kits to use for each step.
- Update to cluster generation reagents and procedure.

Content

1 Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

NOTE Agilent cannot guarantee the SureSelect Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols to process samples for enrichment.

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing RNA on a vortex mixer. Instead, gently tap the tube with your finger to mix the sample.
- Avoid repeated freeze-thaw cycles of stock and diluted RNA solutions.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

Table 1 Required Reagents for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
Agilent DNA 1000 Kit	Agilent p/n 5067-1504
Agilent High Sensitivity DNA Kit	Agilent p/n 5067-4626
Herculase II Fusion DNA Polymerase (includes dNTP mix and 5x Buffer) 200 reactions 400 reactions	Agilent p/n 600677 p/n 600679
AffinityScript Multiple Temperature Reverse Transcriptase 10 reactions 50 reactions 200 reactions	Agilent p/n 600105 p/n 600107 p/n 600109
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Applied Biosystems p/n 4389764
Agencourt AMPure XP Kit 5 mL 60 mL 450 mL	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
Illumina mRNA-Seq Prep Kit	Illumina Cat #RS-100-0801
Illumina Multiplexing Sample Preparation Oligonucleotide Kit (contains Index Adapter mix, InPE1.0 PCR primer, and Index-specific PCR primers)	Illumina Cat # PE-400-1001
Buffer EB (10mM Tris-Cl, ph 8.5)	Qiagen p/n 19086
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
3 M NaOAc, pH 5.2	
Distilled water	

Table 2 Required Reagents for Cluster Generation and Sequencing

Description	Vendor and part number
Illumina Cluster Generation Kit (depending on your instrument and setup)	
TruSeq PE Cluster Kit v5–CS–GA	Illumina p/n PE-203-5001
TruSeq PE Cluster Kit v2–cBot–HS	Illumina p/n PE-401-2001
TruSeq PE Cluster Kit v2.5–cBot–HS	Illumina p/n PE-401-2510
PhiX Control Kit V2 (for HiSeq 2000)	Illumina p/n CT-901-2001
Illumina Sequencing Kit (depending on your instrument and setup)	
TruSeq SBS Kit v5–GA (36-cycle)	Illumina p/n FC-104-5001
TruSeq SBS Kit–HS (50 cycle)	Illumina p/n FC-401-1002

Table 3 Agilent SureSelect RNA Capture Enrichment System Kits (select one)

Reactions	Up to 0.2 Mb (Level MP1)	Up to 0.5 Mb (Level MP2)	Up to 1.5 Mb (Level MP3)	Up to 3.0 Mb (Level MP4)	Up to 6.0 Mb (Level MP0)	Human Kinome
5						G7580A*
10	G7581B*	G7582B*	G7583B*	G7584B*	G7585B*	G7580B*
25	G7581C*	G7582C*	G7583C*	G7584C*	G7585C*	G7580C*
50	G7581D*	G7582D*	G7583D*	G7584D*	G7585D*	G7580D*
100	G7581E*	G7582E*	G7583E*	G7584E*	G7585E*	G7580E*
250	G7581F*	G7582F*	G7583F*	G7584F*	G7585F*	G7580F*
500	G7581G*	G7582G*	G7583G*	G7584G*	G7585G*	G7580G*
1,000	G7581H*	G7582H*	G7583H*	G7584H*	G7585H*	G7580H*
2,000	G7581J*	G7582J*	G7583J*	G7584J*	G7585J*	G7580J*
5,000	G7581K*	G7582K*	G7583K*	G7584K*	G7585K*	G7580K*
10,000	G7581L*	G7582L*	G7583L*	G7584L*	G7585L*	G7580L*

* Order option 001 and 012.

Table 4 Required Reagents for Hybridization

Description	Vendor and part number
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
Dynabeads MyOne Streptavidin T1 2 mL 10 mL 100 mL	Invitrogen Cat #656-01 Cat #656-02 Cat #656-03

Required Equipment

Table 5 Required Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
Agilent 2100 Bioanalyzer	Agilent p/n G2938C
Nuclease-free 1.5 mL microfuge tubes (sustainable at 95°C)	Ambion p/n AM12400 or equivalent
Thermal cycler	Applied Biosystems Veriti Thermal Cycler, BioRad (MJ Research) DNA Engine PTC-200, or equivalent
Nuclease-free 0.2 mL PCR tubes, thin-walled	Eppendorf p/n 951010006 or equivalent
Microcentrifuge	Eppendorf Microcentrifuge Model 5417C or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vacuum concentrator	Savant SpeedVac or equivalent
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vortex mixer	
Heat block at 37°C	

Table 6 Required Equipment for Hybridization

Description	Vendor and part number
Mx3000P/Mx3005P 96-well tube plates	Agilent p/n 410088 or equivalent
Mx3000P/Mx3005P optical strip caps	Agilent p/n 401425 or equivalent
MicroAmp Clear Adhesive Film	Applied Biosystems p/n 4306311 or equivalent
BD Clay Adams Nutator Mixer	BD Diagnostics p/n 421105 or equivalent
Dynal DynaMag-2 magnetic stand	Invitrogen p/n 123-21D or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Pipet-Light Multichannel Pipette, 12 channels	Rainin p/n L12-20 or equivalent
Sterile, nuclease-free aerosol barrier pipette tips	
Thermal cycler	Applied Biosystems Veriti Thermal Cycler, BioRad (MJ Research) DNA Engine PTC-200, or equivalent
Timer	
Vortex mixer	
Water bath or heat block set to 65°C	

Optional Equipment

Table 7 Optional Equipment for Hybridization

Description	Vendor and part number
Tube-strip capping tool	Agilent p/n 410099

2 Sample Preparation

This section contains instructions for prepped library production specific to the Illumina -read sequencing platform. It is intended for use with the Illumina mRNA-Seq Prep Kit (p/n RS-100-0801).

Refer to the Illumina protocol *mRNA Sequencing Sample Preparation Guide* (p/n 1004898 Rev. D) for more information.

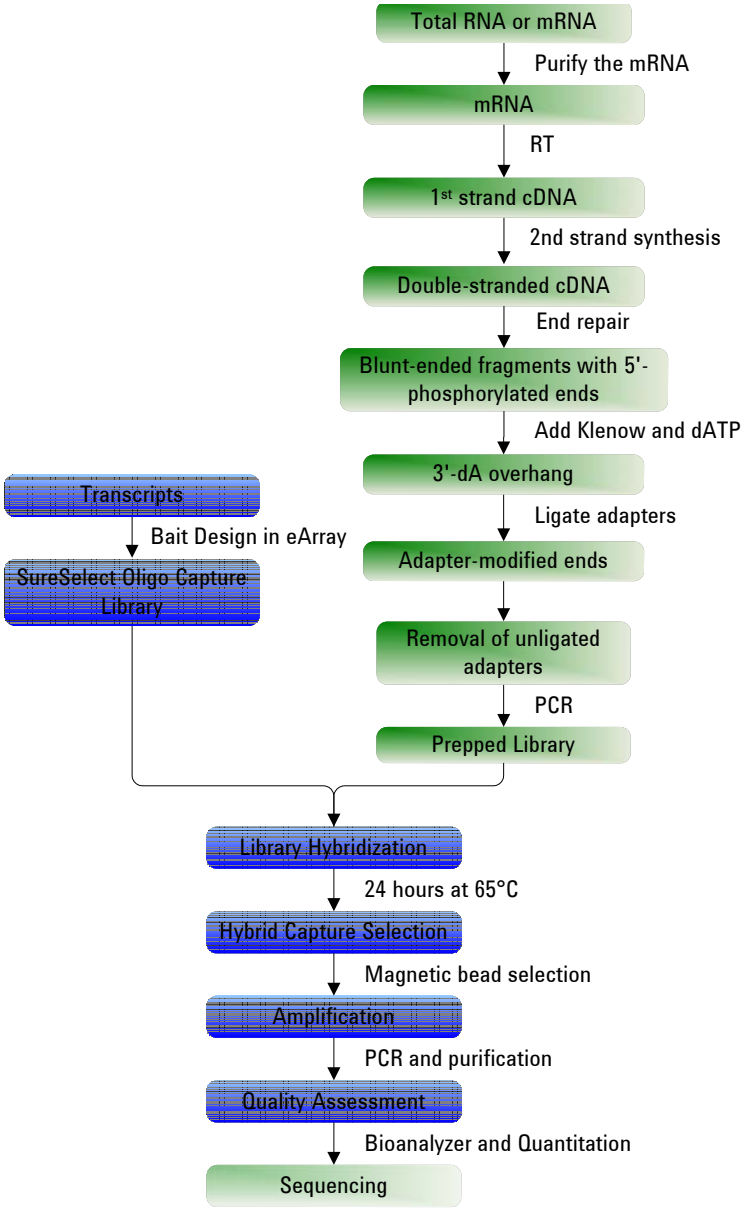

Figure 1 Overall sequencing sample preparation workflow.

Table 8 Overview and time requirements

Step	Time
Illumina Prepped library Production	2 days
Library Hybridization	25 hours (optional 72 hours)
Bead preparation	30 minutes
Capture Selection and Washing	2 hours
DNA purification	30 minutes
Post-Hybridization Amplification	1 hour
PCR purification	30 minutes
Bioanalyzer QC	1 hour

Step 1. Purify the mRNA

Refer to the Illumina protocol *mRNA Sequencing Sample Preparation Guide* (p/n 1004898 Rev. D) for:

- sample prep workflow
- RNA input recommendations
- the use of Stratagene Universal Human Reference (UHR) total RNA as positive control
- best practices for using the magnetic stand

You can start the protocol with 100 ng of mRNA. If so, start at "Step 2. Fragment the RNA" on page 20.

Use reagents from the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801):

- Sera-Mag Magnetic Oligo(dT) Beads
- Bead Binding Buffer
- Bead Washing Buffer
- Ultrapure Water
- 10 mM Tris Buffer

NOTE Use only High quality RNA as the starting material. Check the RNA integrity after isolation, using the Agilent Bioanalyzer 2100. Make sure the RNA Integrity Number (RIN) is greater than 8.

1. Preheat one heat block to 65°C and the other heat block to 80°C.
2. Dilute the 1 to 10 µg of total RNA with nuclease-free water to 50 µl in a 1.5 mL RNase-free non-sticky tube.
3. Heat the sample in a preheated heat block at 65°C for 5 minutes to disrupt the secondary structures, and then place the tube on ice.
4. Put 15 µL of Sera-Mag oligo(dT) beads into a 1.5 mL RNase-free non-sticky tube.
5. Wash the beads two times with 100 µL of Bead Binding Buffer, then remove the supernatant.
6. Resuspend the beads in 50 µL of Bead Binding Buffer and add the 50 µL of total RNA sample from step 3.
7. Rotate the tube from step 6 in a rotator at room temperature for 5 minutes.

8 While the tube incubates, put 50 µL of Bead Binding Buffer into a fresh 1.5 mL RNase-free non-sticky tube.

9 After the 5 minutes incubation, wash the beads from step 7 twice with 200 µL of Bead Washing Buffer, then remove the supernatant.

10 Add 50 µL of 10 mM Tris Buffer to the beads and then heat in the preheated heat block at 80°C for 2 minutes to elute the mRNA from the beads.

11 Immediately put the tube on the magnet stand, and transfer the supernatant (mRNA) to the tube from step 8. Do not discard the used beads.

12 Place the samples aside and wash the beads twice with 200 µL of Bead Washing Buffer.

13 Heat the samples in the preheated heat block at 65°C for 5 minutes to disrupt the secondary structures and then place the samples on ice.

14 Add the iced 100 µL of the mRNA sample from step 13 to the washed beads and rotate it at room temperature for 5 minutes, then remove the supernatant.

15 Wash the beads twice with 200 µL of Bead Washing Buffer and remove the supernatant.

16 Add 17 µL of 10 mM Tris Buffer to the beads and heat in the preheated heat block at 80°C for 2 minutes to elute the mRNA from the beads.

17 Immediately put the tube on the magnet stand and then transfer the supernatant (mRNA) to a fresh 200 µL thin-wall PCR tube.

The resulting amount of mRNA is approximately 16 µL.

Step 2. Fragment the RNA

Use reagents from the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801):

- 5X Fragmentation Buffer
- Fragmentation Stop Solution
- Glycogen
- Ultrapure Water

1 Preheat a PCR thermal cycler to 94°C.

2 Add the components in Table 9 to a 200 µL thin wall PCR tube.

Table 9 Fragmentation Buffer Mix

Component	Volume
5X Fragmentation Buffer*	4 µL
mRNA	16 µL
Total	**20 µL**

* Included in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801) (p/n RS-100-0801).

3 Incubate the tube in a preheated PCR thermal cycler at 94°C for exactly 5 minutes.

4 Add 2 µL of Fragmentation Stop Solution.

5 Put the tube on ice.

6 Transfer the solution to a 1.5 mL RNase-free non-sticky tube.

7 Add the components in Table 10 to the tube and incubate at -80°C for 30 minutes or overnight as desired.

Table 10

Component	Volume
3 M NaOAC, pH 5.2	2 µL
Glycogen*	2 µL
100% ethanol	60 µL

* Included in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801) (p/n RS-100-0801).

Stopping Point You can safely stop the protocol here. Store the samples at -15°C to -25°C. Do not stop the protocol at any other point while the sample is RNA.

8 Spin the tube in a microcentrifuge at 14,000 rpm (20,200 relative centrifugal force) for 25 minutes at 4°C.

9 Carefully pipette off the ethanol without dislodging the RNA pellet.

The RNA pellets are small and almost colorless. To avoid dislodging the pellets, remove the ethanol in several steps. Remove 90% at each step and switch to smaller pipette tips for each step.

10 Without disturbing the pellet, wash the pellet with 300 µL of 70% ethanol.

11 Spin the pellet in a centrifuge and carefully pipette out the 70% ethanol.

12 Air dry the pellet for 10 minutes at room temperature.

13 Resuspend the RNA in 11.1 µL of RNase-free water.

Step 3. Synthesize first strand cDNA

Use reagents from the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801) and the AffinityScript Multiple Temperature Reverse Transcriptase (Agilent).

1 Add the components in Table 11 to a 200µL thin wall PCR tube:

Table 11 Random Primer Mix

Component	Volume
Random Primers*	1 µL
mRNA	11.1 µL
Total	**12.1 µL**

* Included in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801)

2 Incubate the sample in a PCR thermal cycler at 65°C for 5 minutes, and then put the tube on ice.

3 Set the PCR thermal cycler to 25°C.

4 Mix the reagents in Table 12 in the order listed in a separate tube. Prepare 10% extra reagent mix if you are preparing multiple samples.

Table 12 Random Primer Mix

Component	Volume for 1 reaction	Volume for 10 reactions (with excess)
10X AffinityScript RT Buffer (clear cap)*	2 µL	22 µL
100 mM DTT (green cap)*	2 µL	22 µL
25 mM dNTPs Mix†	0.4 µL	4.4 µL
RNaseOUT Ribonuclease Inhibitor†	0.5 µL	5.5 µL
Nuclease-free water	1 µL	11 µL
Total	**5.9 µL**	**64.9 µL**

* Included with the AffinityScript Multiple Temperature Reverse Transcriptase (Agilent).

† Included in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801).

5 Add 5.9 µL of mixture to the PCR tube and mix well.

6 Heat the sample in the preheated PCR thermal cycler at 25°C for 2 minutes.

7 Add 2 µL of AffinityScript Multi-Temp RT (purple cap) to the sample and incubate the sample in a thermal cycler with the program listed in Table 13.

Table 13 PCR program

Temperature	Time
25°C	10 minutes
42°C	50 minutes
70°C	15 minutes
4°C	Hold

8 Put the tube on ice.

Step 4. Synthesize second strand cDNA

Use reagents from mRNA-Seq Prep Kit (Illumina p/n RS-100-0801).

1 Preheat a PCR thermal cycler to 16°C.

2 Add 62.8 µL of Ultrapure Water to the first strand cDNA synthesis mix.

3 Add the reagents the in Table 14 to the mix:

Table 14

Component	Volume
Gex Second-Strand Buffer*	10 µL
25 mM dNTPs Mix*	1.2 µL

* Included in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801).

4 Mix well and incubate on ice for 5 minutes or until well-chilled.

5 Add the reagents in Table 15 to the mix:

Table 15

Component	Volume
RNaseH*	1 µL
DNA Polymerase I*	5 µL

* Included in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801).

6 Mix well and incubate at 16°C in a thermal cycler for 2.5 hours.

Step 5. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 180 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the cDNA library (~100 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 50 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove approximately 50 µL of the supernatant to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 6. Repair the ends

To process multiple samples, prepare master mixes with overage at each step, without the cDNA sample. Master mixes for preparation of 12 samples (including excess) are shown in each table as an example.

Prepare the master mix on ice.

Use the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801).

1 Preheat one heat block to 20°C and the other heat block to 37°C.

2 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 16. Mix well by gently pipetting up and down.

3 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 16. Mix well on a vortex mixer.
   b Add 50 µL of the reaction mix to each well or tube.
   c Add 50 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

4 Incubate in a thermal cycler for 30 minutes at 20°C. Do not use a heated lid.

Table 16 End Repair Mix*

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
cDNA sample	50 µL	
Ultrapure Water	27.4 µL	342.5 µL
10X End Repair Buffer	10 µL	125 µL
25 mM dNTPs Mix	1.6 µL	20 µL
T4 DNA Polymerase	5 µL	62.5 µL
Klenow DNA Polymerase	1 µL	12.5 µL
T4 Polynucleotide Kinase	5 µL	62.5 µL
Total Volume	**100 µL**	**625 µL (50 µL/sample)**

* Included in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801).

Step 7. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 180 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the end-repaired cDNA library (~100 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 32 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

Remove the supernatant (~32 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 8. Add 'A' Bases to the 3' end of the cDNA fragments

Use the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801).

1 For 1 library (prepare on ice):

- In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 17. Mix well by gently pipetting up and down.

2 For multiple libraries (prepare on ice):

a Prepare the reaction mix in Table 17. Mix well on a vortex mixer.

b Add 18 µL of the reaction mix to each well or tube.

c Add 32 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 17 Adding "A" Bases*

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
cDNA sample	32 µL	
10X A-Tailing Buffer	5 µL	62.5 µL
1 mM dATP	10 µL	125 µL
Klenow Exo -	3 µL	37.5 µL
Total Volume	**50 µL**	**225 µL (18 µL/sample)**

* Included in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801).

3 Incubate in a thermal cycler for 30 minutes at 37°C.

Do not use a heated lid.

Step 9. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the A-tailed cDNA library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 18 µL of nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~18 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.
13. Proceed immediately to the next step, "Step 10. Ligate the paired-end adapter".

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 10. Ligate the paired-end adapter

Use the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801) and the Multiplexing Sample Preparation Oligonucleotide Kit (Illumina p/n PE-400-1001).

1 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 18.Use the Illumina mRNA-Seq Prep Kit (p/n RS-100-0801). Mix well by gently pipetting up and down.

2 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 18. Use the Illumina mRNA-Seq Prep Kit (p/n RS-100-0801). Mix well on a vortex mixer.
   b Add 32 µL of the reaction mix to each well or tube.
   c Add 18 µL of each cDNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 18 Ligation master mix

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
cDNA sample	18 µL	
2X Rapid T4 DNA Ligase Buffer	25 µL	312.5 µL
Index PE Adapter Oligo Mix*	6 µL	75 µL
Rapid T4 DNA Ligase	1 µL	12.5 µL
Total Volume	**50 µL**	**400.0 µL (32 µL/sample)**

* Included in the Multiplexing Sample Preparation Oligonucleotide Kit (Illumina p/n PE-400-1001).

3 Incubate for 15 minutes at 20°C on a thermal cycler. Do not use a heated lid.

Step 11. Purify the sample with Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the ligated cDNA (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 52 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~52 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 12. Amplify adapter-ligated library

This step uses PCR to selectively enrich those cDNA fragments that have adapter molecules on both ends, and to amplify the amount of cDNA in the library. The PCR is done with two primers that anneal to the ends of the adapters. Ten to fourteen cycles of PCR are used.

CAUTION This protocol was optimized to minimize PCR-based bias in the library preparation. While most library preparations yield enough cDNA (100 ng) for at least a single hybridization, poor quality RNA samples or other factors can affect yield.

Use reagents in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801), Multiplexing Sample Preparation Oligonucleotide Kit (Illumina p/n PE-400-1001) and SureSelect Target Enrichment Kit Indexing Hyb Module Box #2.

1 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 19. Mix well by gently pipetting up and down.

2 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 19. Mix well on a vortex mixer.
   b Add 25 µL of the reaction mix to each well or tube.
   c Add 25 µL of each cDNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 19 PCR Components*

Reagent	Volume for 1 Library	Volume for 12 Libraries
cDNA	25 µL	
Ultrapure Water	12 µL	150.0 µL
5X Phusion Buffer	10 µL	125 µL
PCR InPE 1.0†	1 µL	12.5 µL
SureSelect GA Indexing Pre Capture PCR Reverse Primer (clear cap)‡	1 µL	12.5 µL
25 mM dNTPs Mix	0.5 µL	6.25 µL
Phusion DNA Polymerase (Finnzymes Oy)	0.5 µL	6.25 µL
Total Volume	**50 µL**	**313 µL (25 µL/reaction)**

* Included in the mRNA-Seq Prep Kit (Illumina p/n RS-100-0801), except where indicated.

† Included in the Multiplexing Sample Preparation Oligonucleotide Kit (Illumina p/n PE-400-1001).

‡ Included in the SureSelect Target Enrichment Kit Indexing Hyb Module Box #2.

3 Amplify using the following PCR program:

Table 20 PCR program

Step	Temperature	Time
Step 1	98°C	30 seconds
Step 2	98°C	10 seconds
Step 3	65°C	30 seconds
Step 4	72°C	30 seconds
Step 5		Repeat Step 2 through Step 4 for a total of 10 to 14 times.
Step 6	72°C	5 minutes
Step 7	4°C	Hold

Step 13. Purify the sample with the Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the amplified library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 14. Assess quality and quantity with Agilent 2100 Bioanalyzer

Use the Bioanalyzer DNA 1000 to assess the quantity, quality and size distribution of the PCR products.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results. Check that the electropherogram shows a distribution with a peak size approximately 250 to 275 bp. Measure the concentration of the library by integrating under the peak.

NOTE A minimum of 100 ng of library is required for hybridization.

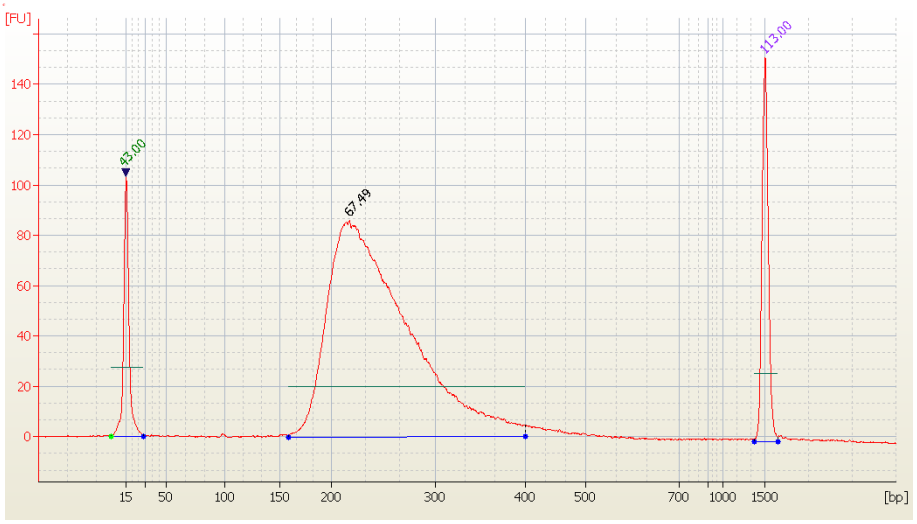

Figure 2 Analysis of amplified prepped library cDNA using a Bioanalyzer DNA 1000 assay. The electropherogram shows a single peak in the size range of approximately 250 bp ± 20%.

3 Hybridization

This chapter describes the steps to combine the prepped library with the hybridization reagents, blocking agents and the SureSelect capture library.

CAUTION The ratio of SureSelect capture library to prepped library is critical for successful capture.

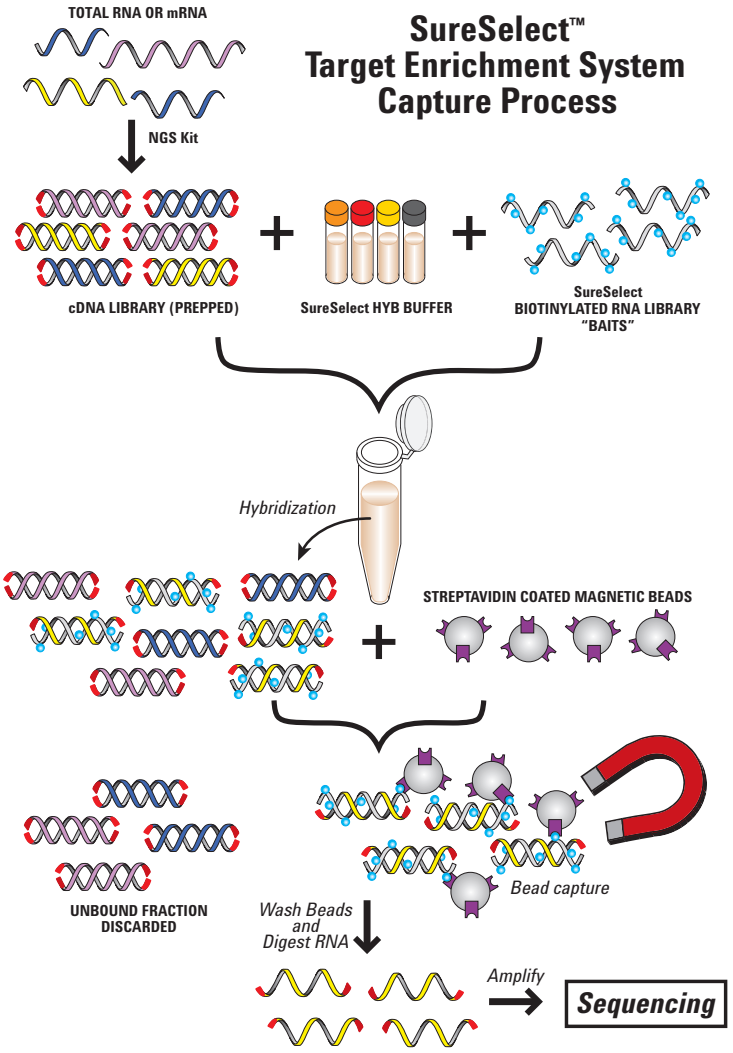

Figure 3 SureSelect RNA Capture Process

Refer to "SureSelect RNA Capture Kit Content" on page 66 for a complete content listing of each SureSelect RNA Capture kit.

CAUTION

You must avoid evaporation from the small volumes of the capture during the 24 hour or greater incubation.

If you want to use a different combination of thermal cycler, lid temperature, plates or strips, and sealing method (strip caps or sealing tape), first test the conditions. Incubate 27 µL of SureSelect Hybridization Buffer (without DNA) at 65°C for 24 hours (or longer, if applicable) as a test. Include buffer in each well that you might use, including those in the center and those on the edges. Check that you do not get extensive evaporation. Evaporation should not exceed 3 to 4 µL.

For a partial list of tested options showing minimal evaporation, refer to "Alternative Capture Equipment Combinations" on page 72.

Step 1. Hybridize the library

The hybridization reaction requires 100 ng of cDNA with a maximum volume of 3.4 µL.

1. If the prepped library concentration is below 30 ng/µL, use a vacuum concentrator to concentrate the sample at ≤ 45°C.
   a. Add the entire volume of prepped library to an Eppendorf tube. Poke one or more holes in the lid with a narrow gauge needle.

      You can also break off the cap, cover with parafilm, and poke a hole in the parafilm.

   b. Completely lyophilize. Use a vacuum concentrator on low heat (less than 45°C) to dehydrate.

   c. Reconstitute with nuclease-free water to bring the final concentration to 30 ng/µL (or greater if sample recovery is of concern). Pipette up and down along the sides of the tube for optimal recovery.

   d. Mix well on a vortex mixer and spin in a microfuge for 1 minute.
2. *Optional.* To test recovery after lyophilization, reconstitute the sample to greater than 30 ng/µL and check the concentration on an Agilent Bioanalyzer DNA 1000 chip. See "Step 14. Assess quality and quantity with Agilent 2100 Bioanalyzer" on page 35. After quantitation, adjust the sample to 30 ng/µL.

   Alternatively, concentrate a 100 ng aliquot at ≤ 45°C down to 3.4 µL. If the sample dries up completely, resuspend in 3.4 µL of water and mix on a vortex mixer. If processing multiple samples, adjust to equivalent volumes before concentrating.
3. Mix the components in Table 21 at room temperature to prepare the hybridization buffer.

Table 21 Hybridization Buffer

Reagent	Volume for 1 capture (µL), includes excess	Volume for 6 captures (µL), includes excess	Volume for 12 captures (µL), includes excess
SureSelect Hyb # 1 (orange cap, or bottle)	25	125	250
SureSelect Hyb # 2 (red cap)	1	5	10
SureSelect Hyb #3 (yellow cap)	10	50	100
SureSelect Hyb # 4 (black cap, or bottle)	13	65	130
Total	**49 (40 µL needed)**	**245 (40 µL needed)**	**490 (40 µL/sample)**

4 If precipitate forms, warm the hybridization buffer at 65°C for 5 minutes.

5 In a PCR plate, strip tubes, or tubes, prepare the SureSelect capture library mix for target enrichment:

   a Keep tubes on ice until step 10.

   b For each sample, add 5 µL of SureSelect capture library.

   c For 1 library, combine 1 µL SureSelect RNase Block (purple cap) with 2 µL nuclease-free water. For multiple libraries, use 1 part SureSelect RNase Block (purple cap) to 2 parts nuclease-free water to make enough mix for 2 µL per capture library, plus excess.

   d Add 2 µL of diluted SureSelect RNase Block (purple cap) to each capture library, and mix by pipetting.

6 Mix the contents in Table 22 to make the correct amount of SureSelect Block mix for the number of samples used.

Table 22 SureSelect Block Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
SureSelect Indexing Block #1 (green cap)	2.5 µL	31.25 µL
SureSelect Block #2 (blue cap)	2.5 µL	31.25 µL
SureSelect GA Indexing Block #3 (brown cap)	0.6 µL	7.5 µL
Total	**5.6 µL**	**70 µL**

7 In a separate PCR plate, prepare the prepped library for target enrichment.

- **a** Add 3.4 µL of 30 ng/µL prepped library to the "B" row in the PCR plate. Put each sample into a separate well.
- **b** Add 5.6 µL of the SureSelect Block Mix to each well in row B.
- **c** Mix by pipetting up and down.
- **d** Seal the wells of row "B" with caps and put the PCR plate in the thermal cycler. Do not heat the Hybridization Buffer or capture library yet, only the prepped library with blockers.
- **e** Run the following thermal cycler program in Table 23.

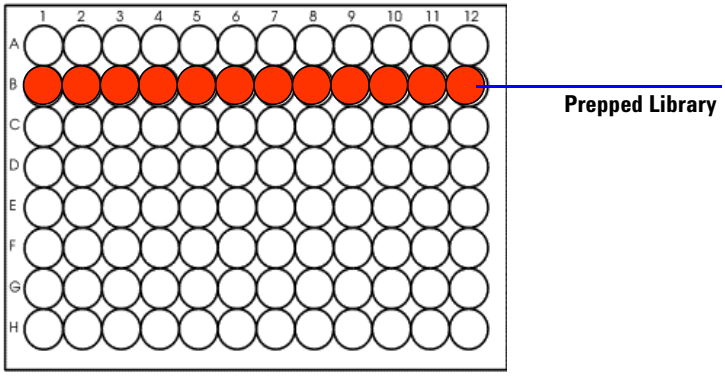

Figure 4 Prepped library shown in red

Table 23 PCR program

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold

8 Use a heated lid on the thermal cycler at 105°C to hold the temperature of the plate on the thermal cycler at 65°C.

CAUTION The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

9 Maintain the plate at 65°C while you load 40 µL of hybridization buffer per well into the "A" row of the PCR plate. The number of wells filled in Row A is the number of libraries prepared.

The example in Figure 5 is for 12 captures.

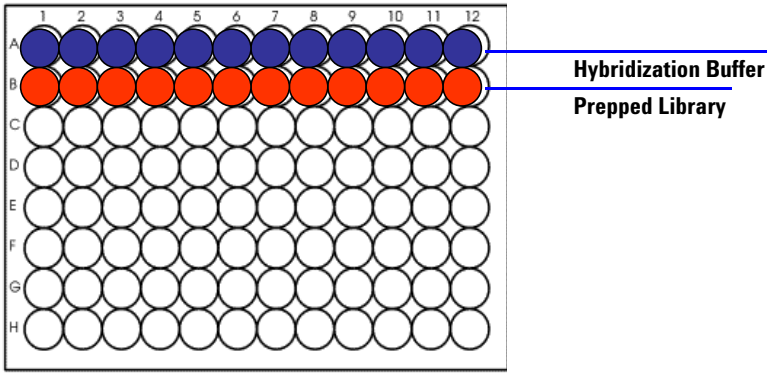

Figure 5 Hybridization buffer shown in blue

Make sure that the plate is at 65°C for a minimum of 5 minutes before you go to step 10.

10 Add the capture library mix from step 5 to the PCR plate:

a Add the capture library mix (7 µL) to the "C" row in the PCR plate.

For multiple samples, use a multi-channel pipette to load the capture library mix into the "C" row in the PCR plate.

Keep the plate at 65°C during this time.

b Seal the wells with strip caps. Use a capping tool to make sure the fit is tight.

c Incubate the samples at 65°C for 2 minutes.

11 Maintain the plate at 65°C while you use a multi-channel pipette to take 13 µL of Hybridization Buffer from the "A" row and add it to the SureSelect capture library mix contained in row "C" of the PCR plate for each sample. (See Figure 6.)

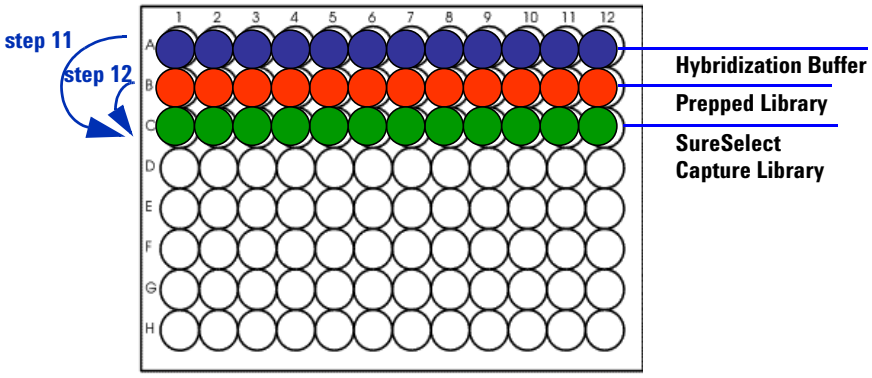

Figure 6 SureSelect Capture Library, or "Baits", shown in Green

12 Maintain the plate at 65°C while you use a multi-channel pipette to transfer the entire contents of each prepped library mix in row "B" to the hybridization solution in row "C". (See Figure 6.) Mix well by slowly pipetting up and down 8 to 10 times.

The hybridization mixture is now 27 to 29 µL, depending on degree of evaporation during the preincubations.

13 Seal the wells with strip caps or double adhesive film. Make sure all wells are completely sealed.

Use new adhesive seals or strip caps. The structural integrity of the seals and caps can be compromised during the previous incubation steps.

CAUTION Wells must be adequately sealed to minimize evaporation, or your results can be negatively impacted.

If you use strip tubes, test for evaporation before you do the first experiment to make sure the tube/capping method is appropriate for the thermal cycler. Check that no more than 3 to 4 µL is lost to evaporation.

14 Incubate the hybridization mixture for 24 hours at 65°C with a heated lid at 105°C.

Samples may be hybridized for up to 72 hours, but when you hybridize at longer periods, do a test to make sure that evaporation is not extensive.

Step 2. Prepare magnetic beads

Use these reagents from the SureSelect Target Enrichment Kit Box #1:

- SureSelect Binding Buffer
- SureSelect Wash Buffer #2

1. Prewarm SureSelect Wash Buffer #2 at 65°C in a circulating water bath for use in "Step 3. Select hybrid capture with SureSelect".
2. Vigorously resuspend the Dynal MyOne Streptavidin T1 (Invitrogen) magnetic beads on a vortex mixer. Dynal beads settle during storage.
3. For each hybridization, add 50 µL Dynal magnetic beads to a 1.5-mL microfuge tube.
4. Wash the beads:
   a. Add 200 µL of SureSelect Binding Buffer.
   b. Mix the beads on a vortex mixer for 5 seconds.
   c. Put the tubes into a magnetic device, such as the Dynal magnetic separator (Invitrogen).
   d. Remove and discard the supernatant.
   e. Repeat step a through step d for a total of 3 washes.
5. Resuspend the beads in 200 µL of SureSelect Binding Buffer.

Step 3. Select hybrid capture with SureSelect

Use these reagents from the SureSelect Target Enrichment Kit Box #1:

- SureSelect Wash Buffer #1
- SureSelect Wash Buffer #2
- SureSelect Elution Buffer
- SureSelect Neutralization Buffer

1. Estimate and record the volume of hybridization that remained after 24 hour incubation.
2. Keep the PCR plate or tubes at 65°C in the PCR machine while you add the hybridization mixture directly from the thermal cycler to the bead solution. Invert the tube to mix 3 to 5 times.

   Excessive evaporation, such as when less than 20 µL remains after hybridization, can indicate suboptimal capture performance. See Table 38 on page 72 for tips to minimize evaporation.
3. Incubate the hybrid-capture/bead solution on a Nutator or equivalent for 30 minutes at room temperature.

   Make sure the sample is properly mixing in the tube.
4. Briefly spin in a centrifuge.
5. Separate the beads and buffer on a Dynal magnetic separator and remove the supernatant.
6. Resuspend the beads in 500 µL of SureSelect Wash Buffer #1 by mixing on a vortex mixer for 5 seconds.
7. Incubate the samples for 15 minutes at room temperature.
8. Separate the beads and buffer on a Dynal magnetic separator and remove the supernatant.

9 Wash the beads:

   a Resuspend the beads in 500 µL of 65°C prewarmed SureSelect Wash Buffer #2 and mix on a vortex mixer for 5 seconds to resuspend the beads.

   b Incubate the samples for 10 minutes at 65°C in a recirculating water bath, heat block or equivalent.

      Do not use a tissue incubator. It cannot properly maintain temperature.

   c Invert the tube to mix. The beads may have settled.

   d Separate the beads and buffer on a Dynal magnetic separator and remove the supernatant.

   e Repeat step a through step d for a total of 3 washes.

      Make sure all of the wash buffer has been removed.

10 Mix the beads in 50 µL of SureSelect Elution Buffer on a vortex mixer for 5 seconds to resuspend the beads.

11 Incubate the samples for 10 minutes at room temperature.

12 Separate the beads and buffer on a Dynal magnetic separator.

13 Use a pipette to transfer the supernatant to a new 1.5-mL microfuge tube.

   The supernatant contains the captured DNA. The beads can now be discarded.

14 Add 50 µL of SureSelect Neutralization Buffer to the captured DNA.

Step 4. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 180 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the 100 µL of cDNA library. Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 0.5 mL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

4 Addition of Index Tags by Post-Hybridization Amplification

This chapter describes the steps to add index tags by amplification, purify, and assess quality and quantity of the libraries, and dilute the sample appropriately for cluster amplification, and pool barcoded samples for multiplexed sequencing.

Step 1. Amplify the sample to add index tags

Use reagents from:

- Herculase II Fusion DNA Polymerase (Agilent)
- SureSelect Target Enrichment Kit Indexing Hyb Module Box #2
- Multiplexing Sample Preparation Oligonucleotide Kit (Illumina p/n PE-400-1001)

CAUTION Do not use amplification enzymes other than Herculase II Fusion DNA Polymerase. Other enzymes have not been validated.

CAUTION This protocol was optimized to minimize PCR-based bias in the library preparation.

To determine the number of cycles needed, do a trial amplification with 12 cycles. If you do not get enough yield for Illumina sequencing, repeat with 14 cycles.

CAUTION To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

Prepare 1 amplification reaction for each hybrid capture. Include a negative no-template control.

1 For 1 library:
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 24, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:
   a Prepare the reaction mix in Table 24, on ice. Mix well on a vortex mixer.
   b Add 36 µL of the reaction mix to each well or tube.
   c Add 1 µL of the appropriate index PCR Primer Index 1 through Index 12 from the Multiplexing Sample Preparation Oligonucleotide Kit (Illumina p/n PE-400-1001) to each well and mix by pipetting.

   Use a different index primer for each sample to be sequenced in the same lane. For low-level multiplexing of fewer than 12 samples, refer to page 53 to select the optimal index tag set.

d Use a pipette to add 14 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples to avoid cross-contamination.

Table 24 Herculase II Master Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Captured DNA	14 µL	
Nuclease-free water	22.5 µL	281.25 µL
5X Herculase II Rxn Buffer (clear cap) *	10 µL	125 µL
100 mM dNTP Mix (green cap)*	0.5 µL	6.25 µL
Herculase II Fusion DNA Polymerase (red cap)*	1 µL	12.5 µL
SureSelect GA Indexing Post Capture PCR (Forward) Primer (orange cap)†	1 µL	12.5 µL
PCR Primer Index 1 through Index 12‡	1 µL	
Total	**50 µL**	**437.5 µL (35 µL/reaction)**

* Included in the Herculase II Fusion DNA Polymerase (Agilent). *Do not use the buffer or dNTP mix from any other kit.*

† Included in the SureSelect Target Enrichment Kit Indexing Hyb Module Box #2.

‡ Use one of the 12 primers included in the Multiplexing Sample Preparation Oligonucleotide Kit (Illumina p/n PE-400-1001).

Some sequencing experiments require the use of fewer than 12 index sequences in a lane with a high cluster density. In such cases, select indexes carefully to ensure optimum base calling and demultiplexing by having different bases at each cycle of the index read. Illumina recommends the following sets of indexes for low-level pooling experiments.

Pool of 2 samples:

- Index #6 GCCAAT
- Index #12 CTTGTA

Pool of 3 samples:

- Index #4 TGACCA
- Index #6 GCCAAT
- Index #12 CTTGTA

Pool of 6 samples:

- Index #2 CGATGT
- Index #4 TGACCA
- Index #5 ACAGTG
- Index #6 GCCAAT
- Index #7 CAGATC
- Index #12 CTTGTA

3 Put the tubes in a thermal cycler and run the program in Table 25.

Table 25 PCR program

Step	Temperature	Time
Step 1	98°C	30 seconds
Step 2	98°C	10 seconds
Step 3	57°C	30 seconds
Step 4	72°C	30 seconds
Step 5		• Repeat Step 2 through Step 4 for a total of 12 to 16 times.
Step 6	72°C	5 minutes
Step 7	4°C	Hold

As with the pre-capture PCR amplification, try to enrich the captured DNA with a minimum of PCR cycles. The use of only half of the captured DNA for amplification lets you adjust the number of cycles by repeating the PCR if needed.

As an alternative, you can prepare one PCR master mix as outlined in Table 24. Split this master mix into three small-scale 10 µL PCR reactions and cycle for 10, 12, 14 or 16 cycles. Clean these PCR reactions using the AMPure XP protocol outlined in "Step 2. Purify the sample using Agencourt AMPure

XP beads" on page 23 with these modifications: use 30 µL of AMPure XP beads and elute with 20 µL of nuclease-free water. Run these cleaned samples on a DNA 1000 chip on the Bioanalyzer, as described in "Step 3. Assess quality and quantity with the Agilent 2100 Bioanalyzer High Sensitivity DNA assay" on page 57.

Use the optimal cycle number to repeat PCR at the 50 µL reaction scale. See Table 26 for approximate number of cycles for a given library size. Results may vary based on library content.

Table 26 Cycle times

Capture Size	Cycles
<0.5 Mb	16 cycles
0.5 to 1.49 Mb	14 cycles
> 1.5 Mb	12 cycles

Step 2. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure beads to a 1.5-mL LoBind tube, and add sample library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol is completely evaporated.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at 4°C for up to a week, or at -20°C for longer periods.

Step 3. Assess quality and quantity with the Agilent 2100 Bioanalyzer High Sensitivity DNA assay

Use a Bioanalyzer High Sensitivity DNA Assay to assess the quality and size range. Note that the concentration of each sample loaded on the chip must be within the linear range of the assay to accurately quantify (5 pg to 500 pg). You may need to dilute your sample accordingly. Refer to the *Agilent High Sensitivity DNA Kit Guide* at http://www.chem.agilent.com/en-US/Search/Library/_layouts/Agilent/PublicationSummary.aspx?whid=59504.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results.

   Determine the concentration of the sample by integration under the peak.

   You can use the High Sensitivity Kit to quantify the amount of sample to be used for Illumina sequencing.

   The linear range of the High Sensitivity kit is 5 pg to 500 pg. If the reading far exceeds 500 pg, dilute and run the Bioanalyzer chip again. If the yield is too low or non-specific peaks are observed in the electropherogram, repeat the PCR with more or fewer cycles. The goal is to minimize cycles, while you produce enough library for the quantification needed for application to the flow cell.
8. Continue to sequencing.

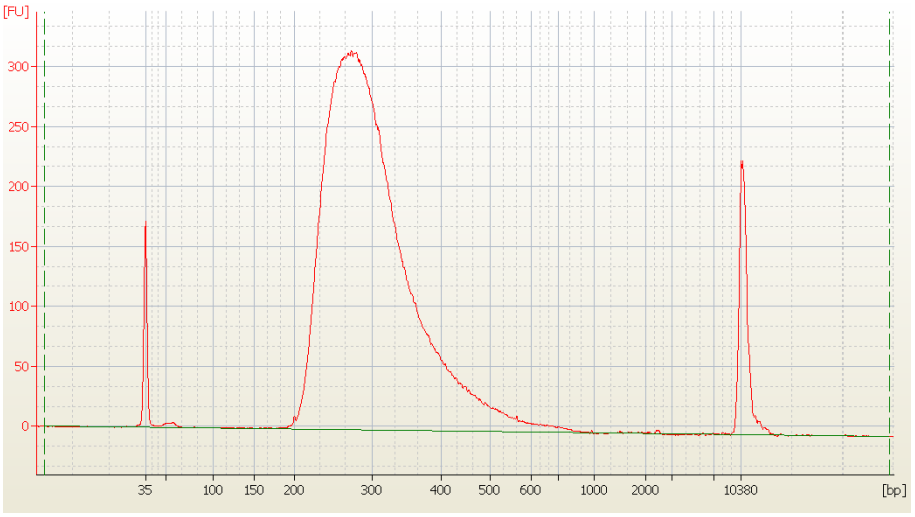

Figure 7 Analysis of Amplified Capture DNA using the High Sensitivity DNA Kit. The electropherogram shows a single peak in the size range of approximately 250 to 300 ± 20% nucleotides.

Step 4. Assess the quantity of each index-tagged library by QPCR

Refer to the protocol that is included with the Agilent QPCR NGS Library Quantification Kit (p/n G4880A) for more details to do this step.

1. Use the Agilent QPCR NGS Library Quantification Kit (for Illumina) to determine the concentration of each index-tagged captured library.
2. Prepare a standard curve using the quantification standard included in the kit, according to the instructions provided in the user guide.
3. Dilute each index-tagged captured library such that it falls within the range of the standard curve.

   Typically this corresponds to approximately a 1:1000 to 1:10,000 dilution of the captured DNA.
4. Prepare the QPCR master mix with Illumina adapter-specific PCR primers according to instructions provided in the kit.
5. Add an aliquot of the master mix to PCR tubes and add template.
6. On a QPCR system, such as the MX3005P, run the thermal profile outlined in the QPCR NGS Library Quantification kit user guide. Use the SYBR Green instrument setting.
7. Use the standard curve to determine the concentration of each unknown index-tagged library, in nM.

   The concentration will be used to accurately pool samples for multiplexed sequencing.

Step 5. Pool samples for Multiplexed Sequencing

1 Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool. For each library, use the formula below to determine the amount of index sample to use.

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)} \text{ where}$$

where *V(f)* is the final desired volume of the pool,

C(f) is the desired final concentration of all the DNA in the pool, for example, 10 nM for the standard Illumina protocol

is the number of index, and

C(i) is the initial concentration of each index sample.

Table 27 shows an example of the amount of 4 index-tagged (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM.

Table 27 Example of index volume calculation for a total volume of 20 µL

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	20 nM	10 nM	4	2.5
Sample 2	20 µL	10 nM	10 nM	4	5
Sample 3	20 µL	17 nM	10 nM	4	2.9
Sample 4	20 µL	25 nM	10 nM	4	2
Low TE					7.6

2 Adjust the final volume of the pooled library to the desired final concentration.

- If the final volume of the combined index-tagged samples is less than the desired final volume, V(f), add Low TE to bring the volume to the desired level.
- If the final volume of the combined index-tagged samples is greater than the final desired volume, V(f), lyophilize and reconstitute to the desired volume.

3 If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at -20°C short term.

4 Proceed to template denaturation and flow cell preparation. Refer to the appropriate Illumina protocol.

Exact library pool dilution and processing can vary based on the flow cell capacity and analysis pipeline versions being used. Refer to the appropriate Illumina user guide for instructions. This protocol has been validated with 36-base paired-end reads. However, read length can be adjusted to achieve the desired research goals.

Step 6. Prepare sample for cluster amplification

In this step you set up cluster amplification.

Conditions are optimized to provide 700K to 900K clusters/mm^2 on the GAIIx and 400K to 600K clusters/mm^2 on a HiSeq instrument.

Genome Analyzer IIx

Use reagents from the TruSeq Cluster Generation Kit appropriate for your instrument:

- HT1 (Hybridization Buffer)
- HP3 (2 N NaOH)

1. Dilute 30 fmol (3µL) of the 10 nM multiplexed sample pool with 16 µL of Buffer EB (10mM Tris-Cl, ph 8.5) for a total volume of 19 µL.
2. Add 1 µL of HP3 (2 N NaOH).
3. Mix the sample briefly on a vortex mixer and pulse centrifuge.
4. Incubate for 5 minutes at room temperature to denature the DNA.
5. Place the sample on ice until you are ready to proceed to final dilution.
6. Dilute 8 µL of denatured DNA with 992 µL of pre-chilled HT1 (Hybridization Buffer) for a final concentration of 12 pM.
7. Mix the sample briefly on a vortex mixer and pulse centrifuge.
8. Continue with cluster generation. Use the TruSeq SBS Kit v5–GA (36-cycle) and the appropriate Illumina multiplexed sequencing protocol.

HiSeq2000 with PhiX spike-in controls

Use reagents from the TruSeq Cluster Generation Kit appropriate for your instrument:

- HT1 (Hybridization Buffer)
- HP3 (2 N NaOH)

Use the PhiX Control Kit V2 (Illumina CT-901-2001) for:

- PhiX Control

1. Prepare a 1:20 dilution of HP3 (2 N NaOH) down to 0.1N NaOH.
2. Prepare 10 nM (10 fmol/µL) dilutions of the amplified capture, based on the Bioanalyzer quantitation.
3. Add 20 fmol (2 µL) of the 10 nM multiplexed sample pool into 8 µL of Buffer EB (10mM Tris-Cl, ph 8.5) to make a 2 nM solution.
4. Add 10 µL of 0.1 N NaOH.
5. Mix the sample briefly on a vortex mixer and pulse centrifuge.
6. Incubate for 5 minutes at room temperature to denature the DNA.
7. Add 980 µL of HT1 (Hybridization Buffer) to the denatured DNA to make 20 pM template solution.
8. Mix the sample briefly on a vortex mixer and pulse centrifuge.
9. Prepare 4 pM template by mixing 200 µL of 20 pM solution with 800 µL of Pre-Chilled HT1 (Hybridization Buffer).

   If densities higher than 400K-600K clusters/mm^2 are desired, prepare a more concentrated sample from the 20 pM solution.
10. Mix the sample briefly on a vortex mixer and pulse centrifuge.
11. Remove 10 µL from solution (1000 µL) to get 990 µL.
12. Add 10 µL of PhiX Control.
13. Mix the sample briefly on a vortex mixer and pulse centrifuge.
14. Dispense 120 µL of diluted denatured sample DNA template and PhiX Control into a strip tube.
15. Place on ice until ready to use.
16. Continue with cluster generation. Use TruSeq SBS Kit–HS (50 cycle) and the appropriate Illumina multiplexed sequencing protocol.

5 Reference

This chapter contains reference information.

SureSelect RNA Capture Kit Content

Each SureSelect RNA Capture Kit contains one or more of each of these individual kits:

Table 28 SureSelect RNA Capture Kit Contents

Product5	Storage Condition	50 Reactions	250 Reactions
SureSelect Target Enrichment Kit Box #1	Room Temperature	5190-1953	5190-1959
SureSelect Target Enrichment Kit Indexing Hyb Module Box #2	-20°C	5190-2332	5190-2333
Capture Library	-80°C		

The content of each of these kits are described in the tables that follow.

Table 29 SureSelect Target Enrichment Kit Box #1

Kit Component
SureSelect Hyb # 1 (orange cap, or bottle)
SureSelect Hyb # 2 (red cap)
SureSelect Hyb # 4 (black cap, or bottle)
3M Sodium Acetate (clear cap)
SureSelect Binding Buffer
SureSelect Wash Buffer #1
SureSelect Wash Buffer #2
SureSelect Elution Buffer
SureSelect Neutralization Buffer

Table 30 SureSelect Target Enrichment Kit Indexing Hyb Module Box #2

Kit Component
SureSelect Hyb #3 (yellow cap)
SureSelect Indexing Block #1 (green cap)
SureSelect Block #2 (blue cap)
SureSelect GA Indexing Block #3 (brown cap)
SureSelect RNase Block (purple cap)
SureSelect GA Indexing Pre Capture PCR Reverse Primer (clear cap)
SureSelect GA Indexing Post Capture PCR (Forward) Primer (orange cap)

Other Reagent Kits Content

These reagents are from kits other than the SureSelect RNA Capture kit. Make sure you use only the reagents listed here.

Table 31 Herculase II Fusion DNA Polymerase (Agilent)

Component
DMSO (green cap)
5X Herculase II Rxn Buffer (clear cap)
100 mM dNTP Mix (green cap)
Herculase II Fusion DNA Polymerase (red cap)

Table 32 AffinityScript Multiple Temperature Reverse Transcriptase (Agilent)

Component
AffinityScript Multi-Temp RT (purple cap)
10X AffinityScript RT Buffer (clear cap)
100 mM DTT (green cap)

Table 33 mRNA-Seq Prep Kit (Illumina p/n RS-100-0801)

Component
Ultrapure Water
10 mM Tris Buffer
5X Fragmentation Buffer
Fragmentation Stop Solution
Glycogen
Random Primers
25 mM dNTPs Mix
RNaseOUT Ribonuclease Inhibitor

Table 33 mRNA-Seq Prep Kit (Illumina p/n RS-100-0801) (continued)

Component
RNaseH
DNA Polymerase I
10X End Repair Buffer
T4 DNA Polymerase
Klenow DNA Polymerase
T4 Polynucleotide Kinase
10X A-Tailing Buffer
1 mM dATP
Klenow Exo -
2X Rapid T4 DNA Ligase Buffer
PE Adapter Oligo Mix
Rapid T4 DNA Ligase
5X Phusion Buffer
PCR Primer PE 2.0
PCR Primer PE 1.0
Phusion DNA Polymerase (Finnzymes Oy)
Bead Binding Buffer
Bead Washing Buffer
Sera-Mag Magnetic Oligo(dT) Beads

Table 34 Multiplexing Sample Preparation Oligonucleotide Kit (Illumina p/n PE-400-1001)

Component
Index PE Adapter Oligo Mix
PCR InPE 1.0
PCR Primer Index 1 through Index 12

Table 35 mRNA-Seq Prep Kit (Illumina p/n RS-100-0801)

Component
Ultrapure Water
10 mM Tris Buffer
5X Fragmentation Buffer
Fragmentation Stop Solution
Glycogen
Random Primers
25 mM dNTPs Mix
RNaseOUT Ribonuclease Inhibitor
RNaseH
DNA Polymerase I
10X End Repair Buffer
T4 DNA Polymerase
Klenow DNA Polymerase
T4 Polynucleotide Kinase
10X A-Tailing Buffer
1 mM dATP
Klenow Exo -
2X Rapid T4 DNA Ligase Buffer
PE Adapter Oligo Mix
Rapid T4 DNA Ligase
5X Phusion Buffer
PCR Primer PE 2.0
PCR Primer PE 1.0
Phusion DNA Polymerase (Finnzymes Oy)
Bead Binding Buffer

Table 35 mRNA-Seq Prep Kit (Illumina p/n RS-100-0801) (continued)

Component
Bead Washing Buffer
Sera-Mag Magnetic Oligo(dT) Beads

Table 36 TruSeq Cluster Generation Kit*

HT1 (Hybridization Buffer)
HP3 (2 N NaOH)

* Use the Illumina Cluster Generation Kit that is appropriate for your instrument and setup. See Table 4 on page 11.

Table 37 PhiX Control Kit V2 (Illumina CT-901-2001)

PhiX Control

Alternative Capture Equipment Combinations

Table 38 lists combinations of thermal cycler, lid temperature, plates or strips, and sealing method (strip caps or sealing tape) other than those used in this protocol that have shown minimal evaporation.

Refer to this list for additional of equipment combination options for hybridization. Note that minimal evaporation is needed to ensure good capture results.

Table 38 Tested options that show minimal evaporation

PCR Machine	Plate/Strips	Cover	Comments
Stratagene Mx3005P QPCR	Mx3000P Strip Tubes (401428)	MX3000P Optical Strip Caps (401425)	Heated Lid
Stratagene Mx3005P QPCR	MicroAmp Optical 96-well reaction plate (N801-0560)	MicroAmp Clear Adhesive Film (4306311)	Heated Lid; ABI compression pad (4312639) Use two layers of film.
ABI GeneAmp 9700	MicroAmp Optical 96-well Reaction Plate (N801-0560)	MicroAmp Caps (8caps/strip) (N801-0535)	Heated Lid
ABI Veriti (4375786)	MicroAmp Optical 96-well Reaction Plate (N801-0560)	MicroAmp Clear Adhesive Film (4306311)	Heated Lid; ABI compression pad (4312639) Use two layers of film.
Eppendorf Mastercycler	Eppendorf 8-Tube PCR Tubes	Attached lids	Lid heating set to 75°C
BioRad (MJ Research) PTC-200	Stratagene strip tubes 410022 (Mx4000)	Stratagene Optical cap 410024 (Mx4000)	Heated Lid
BioRad (MJ Research) PTC-200	Stratagene strip tubes 410022 (Mx4000)	Stratagene Optical cap 401425 (Mx3000/3005)	Heated Lid
BioRad (MJ Research) PTC-200	Stratagene 96-well Plate 410088 (Mx3000/3005)	Stratagene Optical cap 401425 (Mx3000/3005)	Heated Lid

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In This Book

This guide contains information to run the SureSelect Target Enrichment for Illumina Paired-End mRNA-Seq Library Prep protocol with the SureSelect RNA Capture for Illumina Paired-End Multiplexed Sequencing.

Version 1.1, May 2011

G7580-90010
Revision A1

Agilent Technologies